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IgM in a human neuropathy related to paraproteinemia binds to a carbohydrate determinant in the myelin-associated glycoprotein and to a ganglioside

(monoclonal antibody/myelin proteins/plasma cell dyscrasia/demyelination)

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ABSTRACT **The IgM in three patients with paraproteinemia and peripheral neuropathy was shown to bind to human myelin-associated glycoprotein (MAG) that had been purified to homogeneity by gel filtration on Sepharose CL-6B. The antigenic determinant reacting with the IgM from all three patients was in the carbohydrate part of the MAG molecule. In addition, the IgM from the same three patients bound to a single ganglioside of human sciatic nerve. The results indicate that the IgM paraproteins in these patients react with a carbohydrate determinant that is shared between MAG and a peripheral nerve ganglioside.**

Some patients producing large amounts of monoclonal IgM due to plasma cell dyscrasia have a demyelinating peripheral neuropathy (1). There is evidence to suggest that the neuropathies are caused by binding of the IgM paraproteins to peripheral nerve antigens (2–9), although this has not been established. Experiments in several laboratories have provided evidence that the monoclonal IgM in some of these patients binds to the myelin-associated glycoprotein (MAG) (10–14). In this paper, it is demonstrated that the monoclonal IgM antibodies in three neuropathy patients of this type bind to human MAG purified to homogeneity by the procedure routinely used in our laboratory for chemical studies on this glycoprotein (15). It is further shown that the antibodies are directed against determinants in the carbohydrate part of the MAG molecule. Experiments are also described, however, which indicate that the IgM antibodies produced by the same patients bind to a ganglioside of human sciatic nerve. The results indicate that there are at least two molecules in peripheral nerve containing the carbohydrate antigen that reacts with the paraproteins produced by these patients. If the monoclonal IgM causes the neuropathy in these patients, both MAG and the ganglioside are candidates to be involved in the dysimmune phenomena. Some of the results reported here have been described in abstract form (13, 16).

MATERIALS AND METHODS

Patients. After informed consent was obtained, four patients with IgM paraproteinemia and peripheral neuropathy were studied at the National Institute of Neurological and Communicative Disorders and Stroke. The patients were studied clinically, electromyographically, and by examination of sural nerve biopsies as described (6). Fractionation and identification of serum monoclonal proteins was performed by high-resolution agarose gel electrophoresis combined with immunofixation using specific antisera against human immunoglobulin heavy chains and κ and λ light chains.

Preparative and Analytical Procedures with MAG. Human and rat MAG were selectively extracted from the purified myelin by the lithium diiodosalicylate/phenol procedure (17) and further purified by gel filtration on Sepharose CL-6B as described (15). Deglycosylation of partially purified MAG obtained by the lithium diiodosalicylate/phenol procedure was carried out with trifluoromethanesulfonic acid (18). The polyclonal rabbit antisera to MAG were prepared and characterized as described (19). The IgG monoclonal antibody (7E10) was prepared from mice immunized with rat MAG (20), and the mouse IgM monoclonal antibody to human MAG was prepared by a similar procedure. Electrophoresis was on $NaDodSO_4$ 10% (wt/vol) polyacrylamide gels (21) that were stained for protein with Coomassie blue or for carbohydrate with periodic acid/Schiff reagents (22). For immunostaining, proteins were transferred to nitrocellulose sheets essentially by the procedure of Towbin *et al.* (23). Immune staining was with appropriate peroxidase-labeled second antibodies and 3,3′-diaminobenzidine as substrate. Immune staining with the patients' sera was achieved with peroxidase-labeled goat anti-human IgM, μ-chain specific, obtained from Cappel Laboratories (Cochcranville, PA). Concanavalin A binding to MAG that had been transferred to nitrocellulose strips was demonstrated with horseradish peroxidase according to the principle utilized by Wood and McLaughlin (24) with blocking and washing procedures similar to those of Glass *et al.* (25). Column-purified MAG was radioiodinated with Bolton–Hunter reagent and immune precipitated with rabbit anti-MAG antiserum followed by goat anti-rabbit IgG as described (26). Double-antibody precipitation of ^{125}I-labeled MAG with the sera from human patients or controls was done by an exactly analogous procedure, except that the incubation included 25 μg of human IgM (Cappel) as carrier and the second antibody was rabbit anti-human IgM (μ-chain specific) made by Dako and obtained from Accurate Chemicals (Westbury, NY).

Preparative and Analytical Procedures with Gangliosides. Ganglioside fractions were isolated from human sciatic nerve and brain by DEAE-Sephadex chromatography, alkali treatment, and Unisil chromatography as described by Ledeen *et al.* (27). GM_1 and GD_{1a} standards were purchased from Supelco (Bellefonte, PA). The amount of ganglioside-sialic acid was measured by the thiobarbituric acid procedure (28).

Binding of human IgM to the ganglioside fraction was determined by an ELISA procedure in the following manner. Gangliosides in 20 μl of methanol were added to wells in Linbro enzyme immunoassay microtitration plates (Flow Laboratories) and the solution was dried by evaporation. The wells were then filled with phosphate-buffered saline at

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Abbreviation: MAG, myelin-associated glycoprotein.
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pH 7.2 (P_i/NaCl) containing 1% bovine serum albumin. After 2 hr, the wells were emptied and 100 μl of the subject's serum diluted in P_i/NaCl containing 1% bovine serum albumin was added. After 5 hr, the wells were washed with P_i/NaCl, and 100 μl of peroxidase-conjugated goat anti-human IgM (Cappel) diluted 1:500 in P_i/NaCl containing 1% bovine serum albumin was added. After an overnight incubation and washing, 200 μl of substrate solution containing 0.1% *o*-phenylenediamine in 0.1 M citrate buffer (pH 4.5) and 0.012% H_2O_2 was added to each well. After 30 min in the dark, the absorbance at 492 nm of each well was read on an MR 580 MicroELISA Auto Reader (Dynatech, Alexandria, VA).

TLC of gangliosides was on aluminum-backed TLC plates (Silica gel 60; Merck, Darmstadt, FRG) obtained from Brinkmann. The plates were developed in chloroform/methanol/0.2% KCl, 50:40:10 (vol/vol), and the gangliosides were detected by resorcinol spray (29). Demonstration of antibody binding to individual gangliosides was done by autoradiography after overlaying the TLC plate with the patient's serum diluted 1:500 followed by radioiodinated goat anti-human IgM. The procedure was essentially that of Magnani *et al.* (30), except that the solvent system indicated above was used and goat anti-human IgM obtained from Cappel was radioiodinated with Bolton–Hunter reagent instead of using radioiodinated $F(ab')_2$ of rabbit anti-mouse IgG.

RESULTS

Patients with IgM Paraproteinemia and Peripheral Neuropathy. The sera from four patients with polyneuropathy, designated A, B, C, and D, were used in these studies, and an IgM κ monoclonal protein was identified in each. No patient had malignancy, multiple myeloma, amyloid, or other associated medical illnesses. Electrophysiological studies on the four patients revealed predominantly demyelinating neuropathies, and sural nerve biopsies showed demyelination with some axonal damage. Three of the patients, A (a 52-year-old male), B (a 56-year-old female), and C (a 58-year-old female), had mixed motor-sensory polyneuropathies of 6–8 years' duration. Patient D (a 59-year-old male) had a severe large-fiber sensory polyneuropathy of 22 years' duration without clinically evident motor weakness. The serum IgM levels expressed as mg/dl were: A, 754; B, 765; C, 462; and D, 1,140 (normal range, 50–320 mg/dl).

Binding of the IgM to Purified MAG. Purified rat and human MAG appeared as single bands after electrophoresis, transfer to nitrocellulose strips, and staining for protein with amido black (Fig. 1*A*). Human MAG consistently exhibited a slightly higher apparent molecular weight than rat MAG. When the nitrocellulose strips were immune stained with serum from patient A with IgM paraproteinemia and peripheral neuropathy, the purified human MAG reacted with the IgM, but there was no reaction with rat MAG (Fig. 1*B*). The same result was obtained with sera from patients B and C. Serum from patient D, who had a higher IgM level than the other three patients, did not react with rat or human MAG and serves as a control to rule out nonspecific binding of IgM in this and other experiments. Fig. 1*C* shows that an IgM monoclonal antibody obtained by standard hybridoma technology from a mouse immunized with human MAG resembled the patients' IgM paraproteins in reacting with human MAG but not with rat MAG.

Reactivity with the purified human MAG was also demonstrated by a double-antibody precipitation using the purified human MAG that had been radioiodinated with Bolton–Hunter reagent as the antigen (Table 1). The maximal radioactivity that could be precipitated by the patients' sera was comparable to that precipitated by known rabbit anti-MAG antiserum. This shows that the component reacting with the IgM cannot be a minor component contaminating the purified MAG.

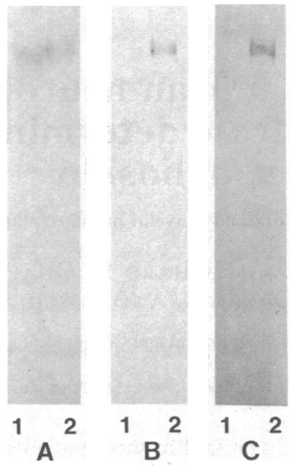

FIG. 1. Immunoblots showing binding of IgM to purified human MAG. (*A–C*) Electroblots of $NaDodSO_4$ gels with purified rat MAG in lane 1 and purified human MAG in lane 2. (*A*) Ten micrograms of purified MAG in each lane; stained for protein with amido black. (*B*) Five-hundred nanograms of purified MAG in each lane; immune stained with a 1:50 dilution of serum from patient A followed by peroxidase-labeled goat anti-human IgM. (*C*) Loaded the same as *B* but stained with the mouse IgM monoclonal antibody produced to human MAG followed by peroxidase-labeled goat anti-mouse IgG and IgM.

Effect of Deglycosylation. To determine if the antigenic site in MAG was in the polypeptide or carbohydrate part of the molecule, isolated MAG was treated with trifluoromethanesulfonic acid. This procedure has been demonstrated to remove all of the carbohydrate from serum-type glycoproteins except for the internal *N*-acetylglucosamine residues by which the oligosaccharides are attached to the polypeptide chains (18). The results of various chemical and immune staining procedures on electroblots of intact and deglycosylated human MAG are shown in Fig. 2. Deglycosylation of MAG decreased its apparent M_r from approximately 100,000 for the intact glycoprotein to about 70,000, as was expected because the molecule is about one-third carbohydrate by weight (15). Staining with periodic acid/Schiff reagents and binding of concanavalin A were greatly reduced in the deglycosylated MAG. The intact MAG and the deglycosylated MAG immune stained both with a polyclonal rabbit antiserum prepared to MAG (19) and with an IgG monoclonal antibody produced by hybridoma technology from a mouse that

Table 1. Immune precipitation of radioiodinated MAG

Serum	Volume, μl	cpm
Control rabbit	5	849
Anti-MAG rabbit	0.5	6,450
Control human	5	1,204
Patient A	2	6,472
Patient B	2	5,950
Patient C	5	3,250*
Patient D	5	902

Increasing amounts of rabbit or patient serum between 0.05 and 5.0 μl were incubated with ^{125}I-labeled human MAG. The volumes shown are the amounts required to give maximal precipitation of radioactive MAG, and the cpm values are averages of duplicate determinations with that volume. The second antibodies were goat anti-rabbit IgG for the rabbit sera and rabbit anti-human IgM for the human sera.

*The binding capacity of this patient was substantially lower than that of patients A and B, and >5 μl was required for maximal precipitation of the radioiodinated MAG.

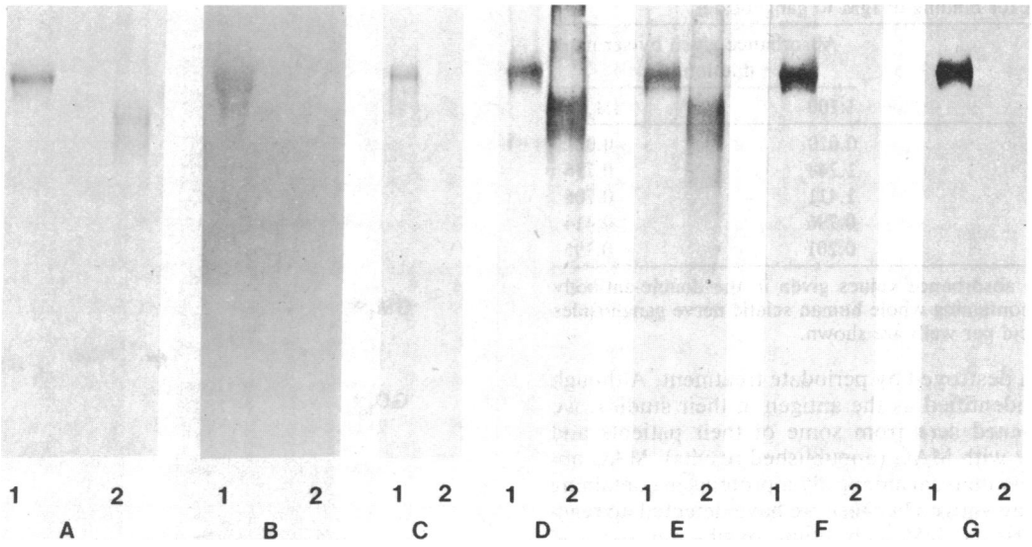

FIG. 2. Immunoblots showing the absence of reactivity of IgM with human MAG after deglycosylation. Part of a $NaDodSO_4$ gel with intact human MAG in lane 1 and deglycosylated human MAG in lane 2 is represented in *A–G*. The MAG in both lanes was from a partially purified preparation obtained by lithium diiodosalicylate/phenol extraction only. (*A* and *B*) The actual gel with staining by Coomassie blue and periodic acid/Schiff reagents, respectively. (*C–G*) Nitrocellulose blots of the gel. (*C*) Stained with concanavalin A and horseradish peroxidase. (*D*) Stained with rabbit polyclonal anti-MAG antiserum (19) (diluted 1:500) followed by peroxidase-labeled goat anti-rabbit IgG. (*E*) Stained with mouse IgG monoclonal antibody 7E10 (20) raised to MAG followed by peroxidase-labeled goat anti-mouse IgG and IgM. (*F*) Stained with serum from patient A (diluted 1:50) followed by peroxidase-labeled goat anti-human IgM. (*G*) Stained with the mouse IgM monoclonal antibody raised to human MAG followed by peroxidase-labeled goat anti-mouse IgG and IgM. The amounts of MAG or deglycosylated MAG were as follows: *A* and *B*, 15 μg; *C*, 220 ng; *D–G*, 500 ng. The MAG and deglycosylated MAG on the various lanes ran as two closely spaced bands representing MAG and a slightly lower molecular weight derivative (dMAG) formed by a protease in purified myelin that has been described (31). The lower molecular weight derivative was more evident in the deglycosylated sample in most cases, probably because it was recovered in somewhat better yield than intact MAG from the deglycosylation step.

had been immunized with isolated rat MAG (20). Although the IgM in a patient with paraproteinemia and peripheral neuropathy stained the intact MAG well, it showed no reactivity with the deglycosylated MAG. The same result was obtained with serum from each of the three patients that showed reactivity with intact MAG (patients A–C). The mouse IgM monoclonal antibody described in Fig. 1*C* also resembled those produced by the patients in reacting with intact MAG but not with the deglycosylated form of the molecule.

Binding of the IgM to a Ganglioside. IgM from the three patients that reacted with MAG also reacted strongly with human sciatic nerve gangliosides in an ELISA (Table 2). Binding of IgM in normal serum or that from patient D was much less. (The elevated absorbance given by serum from patient D in comparison to normal serum is not due to simply nonspecific binding as a result of the high IgM level. Unpublished TLC overlay experiments indicate that this patient's IgM binds to a different ganglioside from the one reacting with the IgM of patients A–C.) The IgM from patients A–C did not react with a ganglioside fraction prepared in the same manner from human brain. The mouse IgM monoclonal antibody raised to human MAG again resembled the patients' IgM in giving a positive reaction to the human sciatic nerve ganglioside fraction (data not shown).

Further evidence for the existence of a ganglioside antigen reacting with the IgM from these three patients with paraproteinemia was obtained by overlaying thin-layer chromatograms of the sciatic nerve ganglioside fraction with the patients' sera followed by radioiodinated goat anti-human IgM. Fig. 3 shows that patient A's IgM bound to a single component on the TLC plate corresponding to one of the fainter gangliosides revealed by resorcinol stain. The IgM from patients B and C bound to the same ganglioside. Overlay experiments also showed that the mouse IgM monoclonal antibody prepared experimentally to human MAG reacted with the same ganglioside, again emphasizing its similarity to the IgM in the neuropathy patients. The results suggest that the human and mouse IgM monoclonal antibodies are reacting with the ganglioside revealed by resorcinol stain (Fig. 3*A*), but the possibility that the antigen is a very minor ganglioside co-chromatographing with the more prominent component has not yet been ruled out. Comparison with known standards showed that the ganglioside reacting with the IgM chromatographed slower than GM_1 and slightly faster than GD_{1a}.

DISCUSSION

Reactivity of the monoclonal IgM in several patients with paraproteinemia and peripheral neuropathy was demonstrated with MAG purified by a procedure developed in our laboratory for obtaining this glycoprotein in homogeneous form for chemical studies (15). IgM binding to the purified MAG was shown both by immunoblotting experiments and by double-antibody precipitation of ^{125}I-labeled MAG. Use of the purified protein confirms and strengthens the evidence now accumulating from several laboratories that MAG is a common antigen reacting with the monoclonal IgM in many patients with paraproteinemia and peripheral neuropathy (10–14). Although all of the results described in this paper were obtained with sera from three patients seen in the National Institutes of Health clinical center, screening of sera sent to us from other laboratories has resulted in the identification of more than 15 patients with paraproteinemia associated with peripheral neuropathy in which the IgM reacts with MAG, including some patients cited in other reports (8, 11).

Because antibodies of the IgM class often react with carbohydrate antigens, the finding that the antigenic site(s) for the paraproteins is in the oligosaccharide moieties of MAG was not unexpected. This observation is consistent with the finding of Liebowitz *et al.* (8) that staining of tissue sections of peripheral nerve with IgM from patients with neuropathy

Table 2. ELISA for binding of IgM to gangliosides

Serum	Absorbance given by serum dilution of 1:100	1:1,000
Normal control	0.020	0.021
Patient A	1.248	0.736
Patient B	1.411	0.708
Patient C	0.796	0.414
Patient D	0.201	0.196

Representative absorbance values given in the double-antibody ELISA in wells containing whole human sciatic nerve gangliosides (10 ng of sialic acid per well) are shown.

of this type was destroyed by periodate treatment. Although MAG was not identified as the antigen in their studies, we have now screened sera from some of their patients and found reactivity with MAG (unpublished results). MAG appears to be highly unusual among glycoproteins in containing this carbohydrate antigen because we have detected no reactivity of the patients' IgM with fetuin, ovalbumin, α-1 acid glycoprotein, whole liver homogenate, or other glycoproteins of peripheral nerve and brain. Furthermore, the antigen possesses a degree of species specificity because we found reactivity with human MAG but not with rat MAG, and others have found reactivity with MAG of several larger mammals but not with mouse, rat, rabbit, or guinea pig MAG (10–12). In our experiments to produce monoclonal antibodies to human MAG in mice by standard hybridoma technology, we have obtained a substantial number of IgM monoclonals that resemble the human paraproteins in reacting with carbohydrate of human MAG but not rat MAG (Figs. 1 and 2; unpublished results). These results suggest that human MAG contains a carbohydrate determinant that is highly immunogenic.

The finding that the IgM from these patients reacts with a ganglioside of peripheral nerve in addition to MAG indicates that there are at least two antigens in the nerve that may be involved in the pathology. It has not been established that the IgM paraproteins in these patients cause the neuropathy by binding to a peripheral nerve antigen, but if they do, either MAG or the ganglioside, or both, could be the significant target antigen. On the other hand, the paraproteinemia in the patients may result from the proliferation of a B-cell clone subsequent to the onset of the neuropathy (9). An autoimmune reaction to the carbohydrate antigen may develop as a secondary response to a preexisting neuropathy, and the chance that the proliferating B cell is one producing anti-MAG (or ganglioside) antibodies would thereby be increased. In either case, the findings indicate that the carbohydrate antigen shared by MAG and the ganglioside is highly immunogenic. Production of antibodies to gangliosides in response to peripheral nerve injury in mice has been described (32). Even if the IgM antibodies do not cause the neuropathy in the patients with paraproteinemia, interaction of the monoclonal antibodies with the carbohydrate antigens may be clinically significant by preventing remyelination.

Other examples of carbohydrate antigens shared by glycoproteins and glycolipids have been described (33–35). The sciatic nerve ganglioside reacting with the IgM in these patients with paraproteinemia was not detected in the ganglioside fraction from brain. Because it chromatographs slower than GM_1 but faster than GD_{1a}, it is probably a mono- or disialo-ganglioside. It is apparently not LM_1, a glucosamine-containing ganglioside of the lacto series, which has been shown to be a major component of peripheral nerve gangliosides (36–38), because that ganglioside migrates slightly faster than GM_1. Work to determine the precise identity of the reactive ganglioside sharing an antigenic determinant with MAG is necessary.

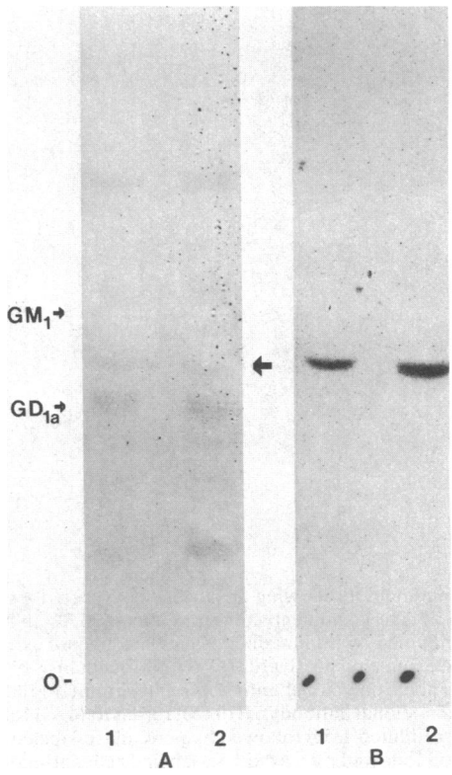

FIG. 3. Binding of patient A's IgM to a ganglioside from human sciatic nerve after separation by TLC. (*A*) Resorcinol-stained gangliosides. (*B*) An autoradiogram of the same chromatogram after overlaying with serum from patient A (diluted 1:500) followed by radioiodinated goat anti-human IgM. Lanes 1 and 2 both contained the same ganglioside fraction from human sciatic nerve representing about 2.5 and 5.0 μg of sialic acid, respectively. The heavy arrow to the right of *A* points to the resorcinol-stained spot corresponding to the ganglioside binding the patient's IgM. The smaller arrows to the left of *A* show the positions where known GM_1 and GD_{1a} standards (not shown) migrated. O, origin.

Radioimmunoassays (26) of human peripheral nerves show that there are only about 0.2 nmol of MAG per g of fresh weight (unpublished result). Estimates from the intensity of resorcinol staining of the reactive ganglioside suggest that its concentration is 10- to 50-fold greater. Therefore, the ganglioside appears to be the quantitatively more prevalent antigen. Thus, when tissue sections of peripheral nerve are immunostained with the IgM paraproteins from these patients, the ganglioside is likely to be the principal antigen visualized. This is supported by the findings that chloroform/methanol extraction of tissue sections eliminated most of the staining (ref. 8; unpublished results), whereas the staining did not appear to be affected by treatment with proteases (8). The presence of the ganglioside antigen is likely to explain the discrepancy between the immune staining of compact myelin produced by the serum from patients with IgM paraproteinemia (refs. 8, 9, 39; unpublished results) and the more selective staining of periaxonal membranes, Schmidt–Lantermann incisures, paranodal membranes, and outer mesaxons in peripheral nerve by rabbit anti-MAG antiserum (40, 41).

The quantitative preponderance of the ganglioside antigen suggests that it may be more significant in the pathology of the disease, although the presence of MAG in critical structures of myelin sheaths (41) indicates that it could also be of prime importance. Indeed, MAG has been shown to be altered early in the pathological changes of central nervous system myelin sheaths in multiple sclerosis (42, 43). Both

MAG and the ganglioside must be considered as potentially causal antigens in the pathogenesis of neuropathy of this type.

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